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SURFACE DISPLAY VECTOR OF SARS VIRUS ANTIGEN AND MICROORGANISMS TRANSFORMED THEREBY

[NAME OF INVENTION]

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[BRIEF DESCRIPTION OF DRAWINGS]

FIG. 1 shows the relations between four antigenic sites (A, B, C, D) of swine transmissible gastro enteritis virus and the spike protein of SARS coronavirus by hydrophilicity plot according to the Kyte-Doolittle method, antigenic index according to the Jameson-wolf method and surface probability plot according to the Emini method.

FIG. 2 shows the relation between the nucleocapsid protein of swine transmissible gastro enteritis virus and the nucleocapsid protein of SARS coronavirus by hydrophilicity plot according to the Kyte-Doolittle method, antigenic index according to the Jameson-wolf method and surface probability plot according to the Emini method.

FIG. 3A is a genetic map of the vector pHCE2LB:pgsA-SARS SA for surface expression comprising the gram negative and gram positive microorganisms as a host according to the present invention, FIG. 3B is a genetic map of pHCE2LB:pgsA-SARS SC according to the present invention and FIG. 3C is a genetic map of pHCE2LB:pgsA-SARS SBC according to the present invention.

FIG. 4A is a genetic map of the vector pHCE2LB:pgsA-SARS NB

according to the present invention.

FIG. 5A is a genetic map of the vector pHCE2LB:pgsA-TGE N1 according to the present invention. FIG. 5B shows fusion proteins of TGEN1 antibody and pgsA in *Lactobacillus* by performing a Western immunoblotting result using SDS-polyacrylamide gel electrophoresis and a specific antibody to TGE N and pgsA. In FIG. 5B, lane 1 is non-transformed *Lactobacillus casei*, and lane 2, 3 are *Lactobacillus casei* transformed with pHCE2LB:pgsA-TGE N1.

FIG. 6A is a genetic map of the vector pHCE2LB:pgsA-PEDN according to the present invention. FIG. 6B shows fusion proteins of PEDN antibody and pgsA in *Lactobacillus* by performing a Western immunoblotting result using SDS-polyacrylamide gel electrophoresis and a specific antibody to PEDN and pgsA. In FIG. 6B, lane 1 is non-transformed *Lactobacillus casei*, and lane 2, 3 are *Lactobacillus casei* transformed with pHCE2LB:pgsA-PEDN.

FIG. 7A and 7B are graphs showing a IgG antibody value to TGEN1 and PEDN antigen protein using ELISA in a serum of each mouse which has been orally and intranasally administered 3 times or twice and boosting administed with the *Lactobacillus casei* strains transformed with pHCE2LB:pgsA-TGEN1 or pHCE2LB:pgsA-PEDN.

[DETAILED DISCLOSURE OF INVENTION]

[OBJECT OF INVENTION]

[TECHNICAL FIELD AND PRIOR ART]

TECHNICAL FIELD

The present invention relates to a vector expressing antigens of SARS on the surface of a microorganism, a microorganism transformed by the vector, and a vaccine for prevention of SARS comprising the transformed microorganism or an extracted and purified substance thereof. More particularly, it relates to a surface expression vector containing a gene encoding antigen proteins of SARS

inducing coronavirus and any one or two or more of genes pgsB, pgsC and pgsA encoding poly-gamma-glutamic acid synthase complex which is a microorganism surface anchoring motif, a microorganism transformed by the vector, and a SARS vaccine comprising the transformed microorganism as an effective ingredient.

BACKGROUND ART

Severe Acute Respiratory Syndrome (SARS) is a new type of an epidemic which has spread all over the world including Hong Kong, Singapore, Canada (Toronto) and so forth since it firstly broke out in November 2002 centering around Guangdong province in China. It shows respiratory symptoms such as fever of 38°C or higher and coughing, dyspnoea, atypical pneumonia. The agent of SARS is known as a mutant pathogenic coronavirus.

Generally, the members of coronavirus family are very large RNA viruses having (+)RNA. The genome is composed of about 29,000 to 31,000 bases and observed as a crown shape under a microscope. It contributes to upper respiratory diseases in human, respiratory, liver, nerves and intestines related diseases in animals. Three groups of coronavirus exist in nature. Among them, group I and group II infect mammals and group III infects birds.

The known coronavirus in nature sometimes induce lung related diseases in persons with weakened immune system or cause severe diseases in animals such as dogs, cats, pigs, mice, birds and the like. They show a very high mutation rate and a high recombination rate of about 25%. It is presumed that such properties cause mutation of original coronavirus, to produce a novel mutant coronavirus (SARS coronavirus), which is propagated from animals to human.

According to World Health Organization (WHO), 7,447 suspected SARS patients in 31 countries have identified since November, 2002 and 551 of them died. The SARS infection danger zone of 2003 include Beijing, Guangdong, Hong Kong, inner Mongolia, Shanxi and Tianjin in China, Singapore, Toronto in

Canada, Taiwan, Ulanbaator in Mongol, Philippines and the like. However, this has a risk to be spread all over the world.

Since the outbreak on 2002, as to SARS coronavirus, a Germany institute for tropical medicine firstly performed decoding of the nucleotide sequence of SARS virus. The research team decoded the nucleotide sequence of a specific genetic part where the amplification by PCR (Polymerase Chain Reaction) can be done. The decoded result was given to Artus GmbH which is a bioengineering company in Germany and used to develop a kit to detect infection of SARS. This kit can determine the infection of SARS virus by amplification of virus gene from a suspected SARS patient.

Thereafter, the whole genome of SARS virus was decoded and up to now, the sequences of more than 12 isolate strains are completely analyzed. The whole sequence of Urbani strain, which is the firstly isolated strain [dubbing the name of the WHO mission doctor who died of SARS, SARS-Cov strain (Rota, PA, Science 108:5952, 2003; GenBank Accession AY278741)] was decoded by a CDC research team of USA. The Canada British Columbia Cancer search center team analyzed the whole sequence of SARS Tor2 virus strain isolated from a patient in Toronto, Canada, on April 12, 2003 (Marra, M.A., Science 108:5953, 2003; GenBank Accession 274119).

Though the two research teams analyzed coronavirus isolated from patients infected with SARS in each different place, the two viruses showed difference in only 15 bases. This suggests that SARS has been induced from the same virus. Also, according to the result of a genomic analysis of SARS coronavirus, it is known that it has the same components forming proteins as those of the existing coronavirus but shows little homology in genome and amino acids by genome. Rat hepatitis virus and turkey bronchitis virus show similarity to SARS coronavirus. However, the correlation of SARS coronavirus and other coronavirus is presented by molecular taxonomic analysis and it is concluded that SARS coronavirus is different from the existing groups.

At present, the detection of SARS coronavirus begins with PCR and the

positive result of the antibody test is determined by ELISA or IFA. The virus isolation is performed by subjecting a subject identified by PCR to a cell culture test and determining the infection of SARS coronavirus.

There is no fundamental method for treating SARS but supplementary supporting therapy. The research on SARS coronavirus, which is an agent of the new epidemic, is in the beginning step and no vaccine for prevention was developed. Diversified researches are being conducted to develop a vaccine for prevention all over the world.

The technology to attach and express a desired protein onto the cell surface of a microorganism is called as cell surface display technology. The cell surface display technology uses surface proteins of microorganisms such as bacteria or yeast as a surface anchoring motif to express a foreign protein on the surface and has an application scope including production of recombinant live vaccine, construction of peptide/antibody library and screening, whole cell absorbent, whole cell biotransformation catalyst and the like. The application scope of this technology is determined by a protein to be expressed on the cell surface. Therefore, the cell surface display technology has tremendous potential of industrial applicability.

For successive cell surface display technology, the surface anchoring motif is the most important. It is the core of this technology to select and develop a motif expressing a foreign protein on the cell surface effectively.

Therefore, in order to select a surface anchoring motif, the following properties should be considered. (1) It should have a secretion signal to help a foreign protein to pass through the cellular inner membrane so that the foreign protein can be transferred to the cell surface. (2) It should have a target signal to help a foreign protein to be stably fixed on the surface of the cellular outer membrane. (3) It can be expressed in a large quantity on the cell surface but does not affect growth of the cell. (4) It has nothing to do with protein size and can express a foreign protein without change in the three-dimensional structure of the protein. However, a surface anchoring motif satisfying the foregoing

requirements has not yet been developed.

The surface anchoring motives which have been known and used so far are largely classified into four types of cell outer membrane proteins, lipoproteins, secretory proteins, surface organ proteins such as flagella protein. In case of gram negative bacteria, proteins existing on the cellular outer membrane such as LamB, PhoE (Charbit *et al.*, J. Immunol., 139:1658, 1987; Agterberg *et al.*, Vaccine, 8:85, 1990), OmpA and the like have been used. Also, lipoproteins such as TraT (Felici *et al.*, J. Mol. Biol., 222:301, 1991), PAL (peptidoglycan associated lipoprotein) (Fuchs *et al.*, Bio/Technology, 9:1369, 1991) and Lpp(Francisco *et al.*, Proc. Natl. Acad. Sci. USA, 489:2713, 1992) have been used. Fimbriae proteins such as FimA or FimH adhesion of tppe 1 fimbriae (Hedegaard *et al.*, Gene, 85:115, 1989), pili proteins such as PapA pilu subunit have been used as a surface anchoring motif to attempt expression of a foreign protein. In addition, it has been reported that ice nucleation protein (Jung *et al.*, Nat. Biotechnol., 16:576, 1998; Jung *et al.*, Enzyme Microb. Technol., 22:348, 1998; Lee *et al.*, Nat. Biotechnol., 18:645, 2000), pullulanase of *Klebsiela oxytoca* (Kornacker *et al.*, Mol. Microl., 4:1101, 1990), IgA protease of *Neiseria* (Klauser *et al.*, EMBO J., 9:1991, 1990), AIDA-1, which is adhesion of *E. coli*, VirG protein of shigella, a fusion protein of Lpp and OmpA may be used as a surface anchoring motif. Upon use of gram positive bacteria, there have been reported that malaria antigen was effectively expressed using *Staphylococcus aureus* derived protein A and FnBPB protein as a surface anchoring motif, a surface coat protein of lactic acid bacteria used in surface expression, and surface proteins of gram positive bacteria such as *Streptococcus pyogenes* derived M6 protein (Medaglini, D et al., Proc. Natl. Acad. Sci. USA., 92:6868, 1995), *Bacillus anthracis* derived S-layer protein EA1, *Bacillus subtilis* CotB and the like were used as a motif.

The present inventors have developed a novel vector for effectively expressing a foreign protein on the cell surface of a microorganism by using poly-gamma glutamic acid synthesizing complex gene (pgsBCA) derived from

Bacillus genus strain as a novel surface anchoring motif and a method for mass-expressing a foreign protein on the surface of a microorganism transformed by the vector (Korean Patent Application No. 10-2001-48373).

Researches have been conducted to stably express a pathogenic antigen or an antigen determining group in bacteria suitable for mass-production by genetic engineering method using the above-listed surface anchoring motives. Particularly, it has been reported that an exogenous immunogen expressed on the surface non-pathogenic bacteria, when being orally administered in the live state, can induce more sustained and stronger immune response, as compared to vaccines using attenuated pathogenic bacteria or viruses. Such induction of immune response is attributable to the adjuvant action of the surface structures of bacteria to increase antigenicity of the foreign protein expressed on the surface and immune response to the live bacteria in the living body. The development of a recombinant live vaccine of non-pathogenic bacteria using this surface expression system has attracted public attention.

Therefore, the present inventors have succeeded in mass-expressing antigens of SARS coronavirus chosen by gene and protein analyses on the surface of a non-pathogenic microorganism, of which food safety is secured, such as lactic acid bacteria by using poly-gamma-glutamic acid synthesizing complex gene (pgsBCA) derived from *Bacillus* genus strain as a surface anchoring motif and developed an economic and stable vaccine to induce production of antibody to SARS coronavirus in blood and mucosal immunization through oral administration of the microorganism.

[OBJECT OF INVENTION]

Therefore, it is an object of the present invention to provide a vector capable of expressing a SARS coronavirus antigen by employing a surface expression system of a microorganism and a microorganism transformed by the vector.

It is another object of the present invention to provide a transformed

microorganism having an antigen of SARS coronavirus expressed on the surface, a vaccine for prevention of SARS comprising a SARS coronavirus antigen extracted from the microorganism or a SARS coronavirus antigen purified from the microorganism as an effective ingredient.

[CONSTITUTION OF INVENTION]

In order to accomplish the above objects, according to the present invention, there is provided a surface expression vector comprising any one or two or more of pgsB, pgsC and pgsA genes encoding poly-gamma-glutamic acid synthase complex and a gene encoding a spike antigen protein or a nucleocapsid antigen protein of SARS coronavirus.

According to the present invention, as the surface antigen protein gene, any gene encoding a spike antigen protein of SARS coronavirus can be used. It is possible to use a spike antigen protein gene of SARS coronavirus alone or as a complex of two or more. Also, the gene encoding the poly-gamma-glutamic acid synthase complex preferably includes pgsA. The spike antigen protein may be SARS SA, SARS SB, SARS SC, or SARS SD and the nucleocapsid antigen protein may be SARS NA or SARS NB.

Also, the present invention provides a microorganism transformed by the expression vector and a method for producing a spike antigen protein or a nucleocapsid antigen protein of SARS coronavirus comprising culturing the microorganism.

The microorganism applicable to the present invention may be any microorganism which does not show toxicity upon application to a living body, or any attenuated microorganism. For example, it can be properly selected from gram negative bacteria, such as *E. coli*, *Salmonella typhi*, *Salmonella typhimurium*, *Vibrio cholerae*, *Mycobacterium bovis*, *Shigella* and the like or gram positive bacteria such as *Bacillus*, *Lactobacillus*, *Lactococcus*, *Staphylococcus*, *Listeria monocytogenes*, *Streptococcus* and the like. Selection of an edible microorganism such as lactic acid bacteria is particularly preferred.

Further, the present invention provides a vaccine for prevention of SARS comprising a microorganism having the antigen protein expressed on the surface, a crude form extracted from cell membrane components of the microorganism which has been broken, or an antigen protein purified from the microorganism as an effective ingredient.

The vaccine according to the present invention can be used as a medicine for prevention of SARS (Severe Acute Respiratory Syndrome) induced by SARS coronavirus.

The vaccine according to the present invention can be taken by oral administration or in food, subcutaneously or intra-peritoneally injected, or administered by the intranasal route.

Up to date, the infection of SARS coronavirus is known to be induced by infection of a respiratory organ by infectious droplets and presumed to occur at the mucosal surface of the respiratory organ. Thus, the protection of infection by mucosal immunity is very important. Since the microorganism expressing an antigen of SARS coronavirus on the surface has an advantage that can more effectively induce antibody formation on a mucous membrane (mucosal response), the vaccine for oral administration or the vaccine for intranasal administration using the transformed microorganism is expected to be more effective than a parenteral vaccine in the protection against SARS coronavirus.

BEST MODE FOR CARRYING OUT THE INVENTION

The Now, the present invention will be explained in further detail by the following examples. It is apparent to those possessing ordinary knowledge in the art that the examples are only for concrete explanation of the present invention and the scope of the present invention is not limited thereto.

Particularly, though genes of an antigenic site in the spike protein of SARS coronavirus and genes of an antigenic site in the nucleocapsid protein of SARS

coronavirus are applied in the following examples, any antigen protein gene may be used alone or as a complex of two or more.

Also, in the following examples, the gene pgsBCA of the cellular outer membrane protein which is involved in synthesis of poly-gamma-glutamic acid is obtained from *Bacillus subtilis* var. chungkookjang (KCTC 0697BP) and used. However, according to the present invention, the gene includes vectors prepared using pgsBCA obtained from all *Bacillus* genus strains producing poly-gamma-glutamic acid or microorganisms transformed with those vectors. For example, preparation of a vector for a vaccine using the pgsBCA gene derived from other strains having homology of 80% or more with the sequence of the pgsBCA gene existing in *Bacillus subtilis* var. chungkookjang and use of the vector are included in the scope of the present invention.

Further, in the following examples, only pgsA of the gene pgsBCA is used to construct a vector for surface expression. However, as can be inferred from indirect examples, use of the whole or a part of the gene pgsBCA to construct a vector for a vaccine is included in the scope of the present invention.

In the following examples, *Salmonella typhi*, which is a gram negative bacterium and *Lactobacillus*, which is a gram positive bacterium are used as a host for the vector. However, it becomes apparent to those skilled in the art that any kind of gram negative bacteria or gram positive bacteria which have been transformed by the method according to the present invention can provide the same results.

In addition, in the following examples, only cases applying a microorganism itself transformed by the vector for a vaccine according to the present invention as a live vaccine to a living body are presented. However, according to the knowledge of the vaccine-related technical field, it is natural to have identical or similar results even when expression proteins (antigen proteins of SARS coronavirus) crudely extracted from the microorganism or purified expression proteins are applied to a living body.

Example 1: Synthesis of antigenic site gene in spike protein of SARS coronavirus

The spike protein of SARS coronavirus is a glycoprotein composed of 1256 amino acids. In case of other coronavirus which have been much examined, the spike protein is mostly inserted into an envelope protein covering the surface of a virus particle to have a structure exposed to the outside. The exposed site and the antigenic site have been intensively studied as a target antigen of a vaccine to induce virus infection and to prevent the infection.

Therefore, in order to select a site capable of showing antigenicity from the 1256 amino acids of the spike protein of SARS coronavirus, the antigenic site was chosen by comparative analysis of proteins and structural comparative analysis with the spike protein of other swine transmissible gastroenteritis (TGE) coronavirus which has been studied for antigenicity and synthesized. Concretely, the antigenic site of the spike protein of swine transmissible gastroenteritis virus is well known as four sites (A, B, C, D) (Enjuanes, L., Virology, 183:225, 1991). The relation between these sites and the spike protein of SARS coronavirus was analyzed by hydrophilicity plot according to the Kyte-Doolittle method, antigenic index according to the Jameson-wolf method and surface probability plot according to the Emini method and SARS SA, SARS SB, SARS SC and SARS SD were selected from the sequence of the spike protein of SARS coronavirus Tor2 isolate (FIG. 1).

Firstly, based on the sequence of the spike protein of SARS coronavirus Tor2 isolate (21492 - 25259 bases, 1255 amino acids), of which the whole sequence had been identified, the 2 to 114 amino acid site which was expected to be an antigenic site was selected and denominated SARS SA, the 375 to 470 amino acid site was selected and denominated SARS SB, the 510 to 596 amino acid site was selected and denominated SARS SC, and the 1117 to 1197 amino acid site was selected and denominated SARS SD. Among these antigenic sites, genes of the SARS SA and SARS SC sites were synthesized.

In order to synthesize a gene corresponding to the 113 length amino acids

denominated SARS SA, PCR was performed using primers of SEQ ID NOs: 1 to 8 to obtain the amplified SARS SA gene of 339bp.

SEQ ID NO: 1: 5'-ggatcctttattttcttattatttcttactctcactagtggtagtgaccttgaccg-3'

SEQ ID NO: 2: 5'-tgagtgtaattaggagcttgaacatcatcaaaagtggtacaacggtcaaggtc- 3'

SEQ ID NO: 3: 5'-aattacactcaacatacttcatctatgcgtggggtttactatcctgatgaaatttttc- 3'

SEQ ID NO: 4: 5' - aaaatggaagaaataaatcctgagttaaataaagagtgtctgaacgaaaaattt-3'

SEQ ID NO: 5: 5'-cttccattttattctaatgttactgggtttcatactattaatcatacgtttggcaac-3'

SEQ ID NO: 6: 5'-ggcagcaaaataaataccatccttaaaaggaatgacagggttgccaaacgtatg-5'

SEQ ID NO: 7: 5'-atttattttgctgccacagagaaatcaaatgttgtccgtggttgggtttttgg-3'

SEQ ID NO: 8: 5'-ggtaccaagcttattacacagactgtgacttgttgttcatggtagaaccaaaaaccc-3'

In order to synthesize a gene corresponding to the 87 length amino acids denominated SARS SC, PCR was performed using primers of SEQ ID NOs: 9 to 14 to obtain the amplified SARS SC gene of 261bp.

SEQ ID NO: 9: 5'-ggatccgtttgtggtccaaaattatctactgaccttattaagaaccagtgtgtcaat-3'

SEQ ID NO: 10: 5'-gaagaaggagttaacacaccagtaccagtgagaccattaaaattaaaattgacacact-3'

SEQ ID NO: 11: 5'-aactccttcttcaaagcgttttcaaccatttcaacaatttggccgtgatgtttctga-3'

SEQ ID NO: 12: 5'-ctaaaatttcagatgttttaggatcacgaacagaatcagtgaaatcagaaacat-3'

SEQ ID NO: 13: 5'-ctgaaattttagacatttcaccttgtgcttttgggggtgtaagtgtaattaca-3'

SEQ ID NO: 14: 5'-ggtaccaagcttattaaacagcaacttcagatgaagcatttgtaccaggtgtaattac-3'

<u>Example 2:</u> Synthesis of antigenic site gene in nucleocapsid protein of SARS coronavirus

The nucleocapsid protein of SARS coronavirus is a protein composed of 422 amino acids. It has been reported that most of the nucleocapsid proteins of other coronavirus on which much research has been conducted serve as an antigen. Such antigenic site has been intensively studied to use a target antigen of a vaccine to prevent the infection of coronavirus.

Therefore, sites capable of showing antigenicity in the amino acids of the nucleocapsid protein of SARS coronavirus was chosen by comparative analysis

of proteins with the nucleocapsid protein of swine transmissible gastroenteritis (TGE) coronavirus and synthesized.

Concretely, the relation between the nucleocapsid protein of swine transmissible gastroenteritis virus and the nucleocapsid protein of SARS coronavirus was analyzed by hydrophilicity plot according to the Kyte-Doolittle method, antigenic index according to the Jameson-wolf method and surface probability plot according to the Emini method and SARS NA and SARS NB were selected from the sequence of the nucleocapsid protein of SARS coronavirus Tor2 isolate (FIG. 2).

Firstly, based on the sequence of the nucleocapsid protein of SARS coronavirus Tor2 isolate (28120 - 29388 bases, 422 amino acids), of which the whole sequence had been identified, the 2 to 157 amino acid site which was expected to be an antigenic site was selected and denominated SARS NA and the 163 to 305 amino acid site was selected and denominated SARS NB. In the present invention, the gene of the SARS NB site was synthesized.

In order to synthesize a gene corresponding to the 143 length amino acids denominated SARS NB, PCR was performed using primers of SEQ ID NOs: 15 to 24 to obtain the amplified SARS NB gene of 429bp.

SEQ ID NO: 15: 5'-ggatcccctcaaggtacaacattgccaaaaggcttctacgcagagggtagccgtgg-3'
SEQ ID NO: 16: 5'-accacgactacgtgatgaagaacgagaagaggcttgactgccgccacggctacc-3'
SEQ ID NO: 17: 5'-cacgtagtcgtggtaattcacgtaattcaactcctggcagcagtcgtggtaat-3'
SEQ ID NO: 18: 5'-gcgagggcagtttcaccaccaccgctagccatacgagcaggagaattaccacga-3'
SEQ ID NO: 191: 5'-gaaactgccctcgcactttgctgcttgaccgtttgaaccagcttgagagcaa-3'
SEQ ID NO: 20: 5'-tagtgacagtttgaccttgttgttgttggcctttaccagaaactttgctctcaa-3'
SEQ ID NO: 21: 5'-caaactgtcactaagaaatctgctgctgaggcatctaaaaagcctcgtcaaaaacgt-3'
SEQ ID NO: 22: 5'-ggaccacgacgccccaaatgcttgagtgacgttgtactgttttgtggcagtacgtttttg-3'
SEQ ID NO: 23: 5'-gggcgtcgtggtccagaacaaacccaaggtaatttcggggaccaagaccttatccgt-3'
SEQ ID NO: 24: 5'-ggtaccaagcttattaaatttgcggccaatgtttgtaatcagtaccttgacggataagg-3'

Example 3: Construction of pHCE2LB:pgsA-SARS SA for surface expression

The surface expression vectors pHCE2LB:pgsA-SARS SA capable of surface expressing the antigenic sites SARS SA in the spike protein of SARS coronavirus were constructed using pgsA of the gene (pgsBCA) of the cellular outer membrane protein derived from *Bacillus* genus strain and participating in the synthesis of poly-gamma-glutamic acid and a gram negative microorganism and a gram positive microorganism as hosts.

Firstly, in order to introduce the antigenic sites SARS SA in the spike protein of SARS coronavirus to a vector for surface expression having the L1 antigen of human papilloma virus expressed with gram negative and gram positive microorganisms as hosts (a vector containing HCE promoter, which is a constantly high expression promoter, pgsA of the gene (pgsBCA) of the cellular outer membrane protein participating in the synthesis of poly-gamma-glutamic acid and HPV L1 in pAT which is a vector for general use for gram negative and gram positive bactera), pHCE2LB:pgsA-HPVL1 (KCTC 10349BP) was digested with *Bam*HI and *Kpn*I. The HPVL1 gene was removed to prepare a vector pHCE2LB:pgsA for surface expression.

The SARS SA antigen genes synthesized in Example 1 were each digested with restriction enzymes *Bam*HI and *Kpn*I and joined to the C-terminal region of the gene pgsA of the cellular outer membrane protein participating in the synthesis of poly-gamma-glutamic acid of the previously prepared surface expression vector pHCE2LB:pgsA in accordance with the translation codon to prepare vectors pHCE2LB:pgsA-SARS SA (FIG. 3A). The gram positive bacterium *Lactobacillus* was transformed with the prepared surface expression vectors pHCE2LB:pgsA-SARS SA and the presence of pHCE2LB:pgsA-SARS SA plasmids in *Lactobacillus* was examined.

<u>**Example 4:**</u> Construction of pHCE2LB:pgsA-SARS SC vector for surface expression

The surface expression vectors pHCE2LB:pgsA-SARS SC capable of surface expressing the antigenic sites SARS SC in the spike protein of SARS

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coronavirus were constructed using pgsA of the gene (pgsBCA) of the cellular outer membrane protein derived from *Bacillus* genus strain and participating in the synthesis of poly-gamma-glutamic acid and a gram negative microorganism and a gram positive microorganism as hosts.

Firstly, the surface expression vector pHCE2LB:pgsA-SARS SA prepared in the Example 3 was used to introduce the antigenic sites SARS SC in the spike protein of SARS coronavirus. pHCE2LB:pgsA-SARS SA was digested with *Bam*HI and *Kpn*I to remove SARS SA gene to prepare a vector pHCE2LB:pgsA for surface expression.

The SARS SC antigen genes synthesized in Example 1 were digested with restriction enzymes *Bam*HI and *Kpn*I and joined to the C-terminal region of the gene pgsA of the cellular outer membrane protein participating in the synthesis of poly-gamma-glutamic acid of the previously prepared surface expression vector pHCE2LB:pgsA in accordance with the translation codon to prepare vectors pHCE2LB:pgsA-SARS SC (FIG. 3B).

The gram positive bacterium *Lactobacillus* was transformed with the prepared surface expression vector pHCE2LB:pgsA-SARS SBC and the presence of pHCE2LB:pgsA-SARS SC plasmid in *Lactobacillus* was examined.

<u>Example 5:</u> Construction of pHCE2LB:pgsA:SARS NB vector for surface expression

The pHCE2LB:pgsA-SARS NB vector capable of surface expressing the antigenic site SARS NB in the nucleocapsid protein of SARS coronavirus was constructed using pgsA of the gene (pgsBCA) of the cellular outer membrane protein derived from *Bacillus* genus strain and participating in the synthesis of poly-gamma-glutamic acid.

Firstly, the surface expression vector pHCE2LB:pgsA-SARS SA prepared in the Example 3 was used to introduce the antigenic sites SARS NB in the spike protein of SARS coronavirus. pHCE2LB:pgsA-SARS SA was digested with *Bam*HI and *Kpn*I to remove SARS SA gene to prepare a vector pHCE2LB:pgsA

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for surface expression.

The SARS NB antigen gene synthesized in the Example 2 was digested with restriction enzymes *Bam*HI and *Kpn*I and joined to the C-terminal of the gene pgsA of the cellular outer membrane protein participating in the synthesis of poly-gamma-glutamic acid of the previously prepared surface expression vector pHCE2LB:pgsA in accordance with the translation codon to prepare a vector pHCE2LB:pgsA-SARS NB (FIG. 4).

The gram positive bacterium *Lactobacillus* was transformed with the prepared surface expression vector pHCE2LB:pgsA-SARS NB and the presence of pHCE2LB:pgsA-SARS NB plasmid in *Lactobacillus* was examined.

INDIRECT EXAMPLE

Using the gene (pgsBCA) of the cellular outer membrane protein derived from *Bacillus* genus strain, which is participated in the synthesis of poly-gamma-glutamic acid, the genes encoding an antigenic sites of a spike antigen protein or a nucleocapsid antigen protein of swine transmissible gastroenteritis virus (TGE) and Porcine Epidemic Diarrhea virus (PED) were selected to prepare vectors containing the said genes for surface expressing thereof. And then, a microorganism was transformed with the prepared the surface expression vector and the presence of said antigenic proteins in the transformed microorganism was examined. Moreover, it was indirectly confirmed by a clinical demonstration that the transformed microorganism can be used as a vaccine for prevention to TGE and PED.

Indirect Example 1: Construction of pHCE2LB:pgsA-TGEN1 for surface expression and Expression of TGE N antibody

Firstly, 1.1 kb of TGE virus genes cloned in pUC8 which is cloning vector used widely (KR 0138395) was used as a template, and PCR was performed using primers of SEQ ID NOs: 25 and 26 to obtain the amplified an antigenic

gene TGE N1 in nucleocapsid antigen protein of swine transmissible gastroenteritis virus (TGE). The amplified gene is 415bp.

SEQ ID NO: 25: 5'-cgggatccgccaaccagggacaacg-3'

SEQ ID NO: 26: 5'-cccaagcttttatggattcattattagc-3'

The above primers of SEQ ID NO: 25 and 26 were constructed so as to be a restriction site of restriction enzymes *Bam*HI and *Kpn*I existed in the surface expression vector pHCE2LB:pgsA.

The amplified TGE N1 genes were digested with restriction enzymes *Bam*HI and *Kpn*I and joined to the C-terminal region of the gene pgsA (the cellular outer membrane protein gene) of pHCE2LB:pgsA vector prepared in Example 3. The prepared pHCE2LB:pgsA- TGE N1 vector was shown in FIG. 5A.

A *Lactobacillus* was transformed with the prepared surface expression vector pHCE2LB:pgsA- TGE N1 and the expression of the antigenic protein pHCE2LB:pgsA- TGE N1 was examined.

The expression of the antigenic sites in the spike antigen of SARS virus fused with the C-terminal of the gene pgsA synthesizing poly-gamma-glutamic acid was induced by transforming *Lactobacillus casei* with pHCE2LB:pgsA-TGE N1, subjecting the transformed strain in MRS medium (*Lactobacillus* MRS, Becton Dickinson and Company Sparks, USA), to a stationary culture and multiplication at 37℃.

The expression of each spike antigen was identified by performing Western immunoblotting using SDS-polyacrylamide gel electrophoresis and a specific antibody to pgsA. The whole cells of *Lactobacillus casei* whose expression is induced concretely were denatured with proteins obtained at the same cell concentration to prepare samples. They were analyzed by SDS-polyacrylamide

gel electrophoresis and the fractionated proteins were transferred to PVDF membrane (polyvinylidene-difluoride membranes, Bio-Rad). The PVDF membrane with the proteins transferred thereon in a blocking buffer solution (50 mM Tris HCl, 5 % skim milk, pH 8.0) was blocked by shaking for 1 hour and reacted with rabbit-derived polyclone primary antibody to pgsA, which have been diluted 1000 times with the blocking buffer solution, for 12 hours.

After completion of the reaction, the membrane was washed with buffer solution and reacted with biotin-binding secondary antibody to rabbit, which have been diluted 1000 times with the blocking buffer solution, for 4 hours. After completion of the reaction, the membrane was washed with buffer solution and reacted with a avidin-biotin reagent for 1 hour, followed by washing. The washed membrane was treated with H_2O_2 and DAB solution as a substrate and a color developing agent to confirm that the specific bonding between the specific antibody to TGE N1 and the fusion protein (FIG. 5B).

In FIG. 5B, lane 1 is non-transformed *Lactobacillus casei*, and lane 2, 3 are *Lactobacillus casei* transformed with pHCE2LB:pgsA-TGE N1. As shown in FIG. 5B, specific fusion proteins pHCE2LB:pgsA-TGE N1 of about 57kDa was confirmed.

<u>Indirect Example 2:</u> Construction of pHCE2LB:pgsA-PEDN for surface expression and Expression of PED N antibody

In order to introduce N antigenic gene of PED virus into pHCE2LB:pgsA prepared in Example 3, 1.3kb of PED virus genes cloned in pUC8 which is cloning vector used widely (KR 0138395) was used as a template, and PCR was performed using primers of SEQ ID NOs: 27 and 28 to obtain the amplified an antigenic gene PED N in nucleocapsid antigen protein of Porcine Epidemic Diarrhea virus (PED). The amplified gene is 1326bp.

SEQ ID NO: 27: 5'-cgggatccgcttctgtcagctttcagg-3'

SEQ ID NO: 28: 5'-cccaagcttttaatttcctgtatcgaaga-3'

The above primers of SEQ ID NO: 27 and 28 were constructed so as to be a restriction site of restriction enzymes *Bam*HI and *Hind*III existed in the surface expression vector pHCE2LB:pgsA.

The amplified PED N genes were digested with restriction enzymes *Bam*HI and *Hind*III and joined to the C-terminal region of the gene pgsA (the cellular outer membrane protein gene) of pHCE2LB:pgsA vector prepared in Example 3. The prepared pHCE2LB:pgsA-PED N vector was shown in FIG. 6A.

A *Lactobacillus* and *E.coli* were transformed with the prepared surface expression vector pHCE2LB:pgsA-PED N and the expression of the antigenic protein pHCE2LB:pgsA- PED N was examined.

The transformed *Lactobacillus* and *E.coli* were cultured to induce expressions thereof by the same procedures as Indirect Example 1, and then it was confirmed that the PED N antigen fused with pgsA was expressed, by performing Western immunoblotting using SDS-polyacrylamide gel electrophoresis and a specific antibody to PED N and pgsA (FIG. 6B).

In FIG. 6B, lane 1 is non-transformed *Lactobacillus casei*, and lane 2, 3 are *Lactobacillus casei* transformed with pHCE2LB:pgsA- PED N. As shown in FIG. 6B, specific fusion proteins pHCE2LB:pgsA-PED N of about 90kDa was confirmed.

<u>Indirect Example 3:</u> Vaccine effects of *Lactobacillus* which TGE virus and PED virus was expressed on surface.

A *Lactobacillus casei* transformed with pHCE2LB:pgsA-TGE N1 and pHCE2LB:pgsA-PEDN prepared in indirect Example 1 and 2 was cultured to induce expressions of each antigen on the surface of *Lactobacillus*. And then, the TGE N1 antigen and PED N antigen fused with pgsA respectively were examined about antigenic features thereof.

The transformed *Lactobacillus* with pHCE2LB:pgsA-TGE N1 or pHCE2LB:pgsA-PEDN was cultured to harvested to be the same cell concentration and washed several times with buffer solution (PBS buffer, pH7.4). 5 X 10^9 *Lactobacillus* cells with the antigen surface expressed were orally administered to a 4-6 week old BALB/c mouse 3 times a day every other day, 3 times a day every other day after 1 week, 3 times a day every other day after 2 weeks, and 3 times a day every other day after 4 weeks, and then a serum of each mouse was taken and examined for IgG antibody value to N antigen protein by Western immunoblotting (FIG 7A and 7B).

As a result, FIG 7A is a graph showing a IgG antibody value to TGE N antigen protein in serum, ▼ means a mouse oral-administered with *Lactobacillus casei* transformed with pHCE2LB:pgsA- TGE N1, ▽ means a mouse intranasal-administered with *Lactobacillus casei* transformed with pHCE2LB:pgsA- TGE N1, ○ means a mouse oral-administered with *Lactobacillus casei* transformed with pHCE2LB, and ● means a mouse oral-administered with *Lactobacillus*.

Furthermore, FIG 7B is a graph showing a IgG antibody value to PEDN antigen protein in serum, ▼ means a mouse oral-administered with *Lactobacillus casei* transformed with pHCE2LB:pgsA- PEDN, ▽ means a mouse intranasal-administered with *Lactobacillus casei* transformed with pHCE2LB:pgsA- PEDN, ○ means a mouse oral-administered with *Lactobacillus casei* transformed with

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pHCE2LB, and ● means a mouse oral-administered with *Lactobacillus*.

As shown in FIG. 7, it was confirmed that IgG antibody value to each N antigen protein is high compared to the control in serum of the mouse administered orally or intranasally with *Lactobacillus casei* transformed with pHCE2LB:pgsA-TGE N1 and pHCE2LB:pgsA-PEDN. Therefore, it was confirmed that the microorganism(*Lactobacillus)* expressed with N antigen protein of TGE and PED is useful as a raw-vaccine.

[EFFECT OF INVENTION]

As described above, the transformed microorganism expressing an antigen protein of SARS inducing coronavirus on their surface according to the present invention and the antigen protein extracted and purified from the microorganism can be used as a vaccine for prevention and treatment of SARS. Particularly, it is advantageously possible to economically produce a vaccine for oral use using the recombinant strain expressing an SARS coronavirus antigen according to the present invention.